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(54) Title: ANTIBODY VARIANTS

(57) Abstract

    This invention relates to an antibody which is a modified version of a therapeutic antibody with affinity for a cell-surface antigen, said antibody having reduced affinity for the antigen compared with the therapeutic antibody as a result of a modification or modifications to the antibody molecule, wherein the antibody is capable of inducing immunological tolerance to the therapeutic antibody. The invention further relates to a method of inducing immunological tolerance to a therapeutic antibody, comprising administering to a patient an antibody which is a modified version of the therapeutic antibody and which has reduced affinity for the antigen as compared with the therapeutic antibody.

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ANTIBODY VARIANTS

5   This invention relates to modified antibodies for inducing
immunological tolerance in human beings or animals.

Antibodies, or immunoglobulins, comprise two heavy chains
linked together by disulphide bonds and two light chains, each light chain
being linked to a respective heavy chain by disulphide bonds. Each heavy
10   chain has at one end a variable domain followed by a number of constant
domains. Each light chain has a variable domain at one end and a
constant domain at its other end, the light chain variable domain being
aligned with the variable domain of the heavy chain and the light chain
constant domain being aligned with the first constant domain of the heavy
15   chain. The constant domains in the light and heavy chains are not
involved directly in binding the antibody to antigen.

The variable domains of each pair of light and heavy chains
form the antigen binding site. The variable domains of the light and heavy
chains have the same general structure; each domain comprises four
20   framework regions, whose sequences are relatively conserved, connected
by three complementarity determining regions (CDRs). The CDRs are held
in close proximity by the framework regions. CDRs from adjacent light and
heavy chain variable domains together contribute to the formation of the
antigen binding site.

25   Background of the Invention

Antibodies directed to specifically chosen antigens have been
used in the treatment of various conditions. For example, Campath-1
monoclonal antibodies (mAb) have been used successfully to induce
remissions in lymphoma and leukemia patients and for the treatment of
30   rheumatoid arthritis and vasculitis. The target antigen, CD52 (also referred

to as CDw52; see e.g. Xia *et al.*, 1991), is a GPI-anchored glycoprotein of lymphocytes and monocytes (and parts of the male reproductive system). It has an exceptionally short peptide sequence of 12 amino acids and a single, complex, N-linked oligosaccharide at Asn3 (Hale et al, 1990; Xia et al, 1991). CD52 is a good target for antibody-mediated killing and is therefore an effective cell surface molecule for various therapeutic regimens in which reduction in lymphocytes is an objective (e.g. removal of cells from donor bone marrow to prevent graft-versus-host disease, treatment of leukemia and lymphoma, and immuno-suppression).

Several rat anti-human CD52 Campath-1 mAb were generated by fusion of the Y3 rat myeloma line with spleen cells from a rat immunized with human T lymphocytes (Hale et al, 1983). Although the clinical effectiveness of rat Campath-1 mAb has been demonstrated regularly, many patients mounted an anti-antibody (antiglobulin) response against the xenogeneic protein that prevented retreatment with the therapeutic antibody. Antibody therapy is often limited by the antiglobulin response. The anti-idiotypic component (anti-Id; directed against the Ab V regions and in particular the Ab-combining site) inhibits the binding of the Ab to its target while both the anti-Id and the anti-isotypic component (directed against the constant regions) act to accelerate antibody clearance. A major concern is the neutralizing effect of the antiglobulin response. As with antiglobulin responses in general, anti-Id responses interfere with the clinical potency of a therapeutic Ab by forming Ab aggregates that are rapidly cleared from the circulation, reducing the chance for interaction with target antigen. Unfortunately, most antiglobulin sera contain anti-Id antibodies. This has been demonstrated for a number of therapeutic mAb and is especially noted after repeated treatments.

To reduce the immunogenicity of the rat IgG2b Campath-1 antibody, YTH34-5, the gene fragments encoding the VL and VH were humanized by "CDR grafting" of the rodent hypervariable regions onto human framework

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regions (Jones et al, 1986; Reichmann et al, 1988). This was carried out by splicing the CDR sequences encoding the rat Campath-1 antibody onto sequence encoding human framework backbone provided by the crystallographically solved myeloma proteins NEW (for the VH) and REI

5 (for the VL). The resulting protein had low antigen-binding titre and modelling of the humanized V-region showed that residue 27 in the VH framework sequence was critical for preserving the loop structure of CDR1. This residue was changed from the residue found in NEW (Ser) back to the rat residue Phe which resulted in restoration of antigen binding. During the

10 modelling, an additional change (NEW residue Ser to the rat residue Thr) was also suggested. However, in functional assays this substitution had no effect on antigen binding, but the double mutant (Ser27 to Phe27 and Ser30 to Thr30) expressed the most protein and therefore was used to produce therapeutic humanized Campath-1 Ab, designated Campath-1H

15 (Reichmann et al, 1988). As many human VH frameworks have threonine at position 30, this change was not considered an additional risk to the antibody's immunogenicity. The humanized VL and VH were then genetically fused with human light chain and heavy chain constant regions, respectively. In summary, the humanized Campath-1 antibody consists of

20 human residues at all positions except those encoding the 3 CDRs of the light chain, the 3 CDRs of the heavy chain, and residues Phe27 and Thr30 in VH of the heavy chain.

In clinical trials, the humanized version (Campath-1H) was found to be much less immunogenic than the rat IgG2b Campath-1 antibody.

25 Humanization reduces the immunogenicity of rodent mAb, although both the idiotype and the allotype of a humanized mAb might still be targets for humoral responses. Sensitization to idiotype has indeed been documented in some allotype-matched recipients of Campath-1H (Isaacs et al, 1992; Lockwood et al, 1993). These responses were revealed by the

30 presence of anti-Id in the patients' sera. One patient generated high-titre

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anti-Id that crossreacted on the entire panel of CD52 mAb. Humanized Campath-1 antibodies are described in EP 0 328 404 the teachings of which are incorporated herein by reference, in their entirety.

One strategy to further reduce the immunogenicity of
5   Campath-1H might be to re-graft the 6 CDR loops onto well-characterized human germline framework regions. The majority of the humanized V regions so far have used rearranged V-genes as acceptor framework sequence. This was the case for Campath-1H as framework sequences from myeloma proteins were used to provide acceptor sequences for both
10   VH and VL. Rearranged V-genes often contain somatic mutations, acquired during the process of affinity maturation. These will be unique to the individual from which the rearranged genes were derived and therefore may be seen as foreign in another individual. However, there is a possibility that regrafting may introduce new idiotypic epitopes, formed by
15   the junctional regions encompassing CDR residues and new framework residues. Furthermore, humanization alone may not solve the problem of anti-Id responses because the human population is outbred and it is unlikely that all patients will be tolerant to a given humanized mAb. Even in antibody constant regions, there are a number of different alleles which
20   carry allotypic markers to which naturally occurring antiglobulin responses can be demonstrated. The problem is more complex for V-region segments, which show a higher degree of variation both in allotype and haplotype in comparison to constant regions.

Another approach is to induce tolerance to the potentially
25   foreign peptides contained within the Campath-1H V-region. We know that the antiglobulin response is itself a B-cell response which is CD4+ T-cell dependent. Isaacs and Waldmann (1994) demonstrated that mice deprived of CD4+ T-cells were unable to respond to a foreign cell-binding mAb (rat anti-mouse CD8 mAb). CD4+ T-cell depletion was carried out by
30   adult thymectomy combined with administration of a depleting CD4 mAb.

In these mice, the response to subsequently administered mAb or SRBC was measured. CD4+ T-cell deficient mice failed to make either an antiglobulin response or an anti-SRBC response, demonstrating that the anti-Ig response, like the anti-SRBC response, is classically CD4+ T-cell

5   dependent. In order to generate T-cell help and to get the appropriate T-cell response, the adminstered Ab must be processed as a protein antigen and presented, presumably in the context of an MHC class II molecule, by a suitable antigen presenting cell. Therefore, two main strategies can be adopted to decrease the immunogenicity of a humanized V-region. (1)

10   We can "silence" the antibody molecule itself, adopting strategies to eliminate any potential T helper epitopes, or (2) we can present all the potential T helper epitopes in a manner that induces tolerance instead of reactivity to those epitopes.

"Silencing" the antibody molecule:

15   a)   In theory, we might be able to silence the antibody itself so that the immune system will not recognize foreign determinants. This would be possible if we could scan the VL and VH amino acid sequences for motifs that could bind to MHC class II molecules. If we could thus identify key residue(s) in a potential class II peptide that were not involved

20   in antibody specificity or affinity, then it/they could be changed by site-directed mutagenesis to residue(s) that did not allow association with class II molecules. T helper peptides are not random, and any protein has only a limited number of peptides capable of binding to MHC class II molecules, and also to T-cell antigen receptors. However, this is not possible at

25   present because class II-binding peptides are not yet characterized to a sufficient degree to be identified by scanning protein sequences. This is in part due to the heterogeneous nature of class II peptides. Naturally processed peptides isolated from MHC class II molecules are generally larger in size, variable in length and have both ragged ends at C- and N-

30   termini in comparison to processed peptides isolated from MHC class I

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molecules. Whereas class I-derived peptides are mostly of uniform length of 8-9 amino acid residues, MHC class II-associated peptides range from 12-24 amino acids (Rudensky et al, 1991; Hunt et al, 1992; Rudensky and Janeway, 1993). Class I-derived peptides have sequence motifs with

5   specific anchor residues in certain positions allowing their side chains to fit in the binding pockets of the peptide-binding groove, and the peptide-binding groove is closed at both ends. In contrast, class II peptides are bound in an extended conformation that projects from both ends of an "open-ended" antigen-binding groove; a prominent non-polar pocket into

10 which an anchoring peptide side chain fits near one end of the binding groove (Brown et al, 1993).

b)   -   Other strategies that might be adopted to "silence" the antibody if we could predict class II peptide-binding motifs. For example, one could include insertion of a protease cleavage site within any potential

15 class II epitope to increase the chance of peptide degredation before they could be presented in the context of class II. Alternatively, insertion of motifs into a V-region such as Gly-Ala repeats may inhibit the degradation of the V-region into peptides that could associate with class II molecules. In one system, it was shown that EBNA1 Gly-Ala repeats generated a cis-

20 acting inhibitory signal that interfered with antigen processing during MHC class I-restricted presentation such that CTL recognition was inhibited (Levitskaya et al, 1995). Although either of these approaches may hold some promise in the future, they again rely on prediction of potential MHC class II peptides from protein sequence of humanized VL and VH regions

25 and are therefore limited by insufficient knowledge regarding consensus motifs for class II peptides.

Inducing tolerance to T helper epitopes

In lieu of sufficient knowledge regarding class II peptide motifs, we have turned our attention toward induction of tolerance to

30 therapeutic antibodies. In 1986, Benjamin et al and Cobbold et al

described an unexpected property of cell-binding mAb: whereas it was possible to induce tolerance to the Fc region (anti-isotype tolerance), the idiotype remained antigenic under equivalent conditions. Moreover, it was relatively easy to induce tolerance to non-cell binding mAb but cell-binding

5   mAb were found to be very immunogenic.

       Isaacs and Waldmann (1994) in a preliminary study used a non-cell-binding "mixed molecule" derivatives of a cell-binding Ab to induce tolerance to the wild-type form. The cell-binding antibody was an anti-CD8 mAb in a mouse model. The non-cell-binding derivatives were made by

10   pairing the relevant L- and H-chains with an irrelevant H- or L-chain, respectively. The relevant H-chain paired with an irrelevant L-chain was obtained by limiting dilution cloning of the original hybridoma that was expressing a myeloma light chain (from the Y3 fusion partner), as well as the specific anti-CD8 H- and L-chains. A variant of the hybridoma that

15   expressed the myeloma L-chain and the specific anti-CD8 H-chain but no anti-CD8 L-chain was obtained. A clone expressing the relevant L-chain only was also obtained in this manner. That clone was then fused to a hybridoma expressing an irrelevant specificity (anti-human CD3) and a variant was selected that expressed the relevant anti-CD8 L-chain with the

20   irrelevant anti-CD3 H-chain. Because proteins are processed into peptides prior to presentation to T-cells, helper peptides from antigen-specific H- and L-chains would be "seen" by T-cells, regardless of their partner chain. However, in this case, there was no advantage in tolerance induction using non-cell-binding mixed molecule derivatives of a therapeutic mAb in vivo

25   compared to an isotype-matched control, suggesting that in the strain of mice used, most (or all) of the helper epitopes were located within the constant region.

       In practice, using these "mixed molecules" of antigen-specific and irrelevant immunoglobulin chains for human therapy would not be

30   feasible because the irrelevant H- and L-chains would carry some helper

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epitopes themselves, thus complicating the ability to achieve tolerance to
the relevant H- and L-chains. Nor would one expect to tolerize those B-
cells which "see" idiotypic determinants formed by the combination of the
relevant H- and L-chains of the antibody.

5   Campath-1 is a cell-binding mAb, and an effective tolerogen
for use with it, such as a non-cell-binding form of the therapeutic mAb
would therefore be advantageous. The same goes for other therapeutic
antibodies which have cell-binding properties, and non-cell-binding variants
thereof.

10 **The Invention**

The invention therefore provides an antibody which is a
modified version of a therapeutic antibody with affinity for a cell-surface
antigen, said antibody having reduced affinity for the antigen compared
with the therapeutic antibody as a result of a modification or modifications

15 to the antibody molecule, wherein the antibody is capable of inducing
immunological tolerance to the therapeutic antibody.

Preferably, the affinity of the antibody according to the
invention for the antigen is reduced to 50% or less of the affinity of the
therapeutic antibody for the antigen. More preferably, the affinity is

20 reduced to 10% or less, or to 1% or less of the affinity of the therapeutic
antibody. The affinity needs to be sufficiently reduced to allow the antibody
according to the invention to act as a tolerogen with respect to the
therapeutic antibody. The term "non-cell-binding variant" is used herein to
refer to antibodies according to the invention, although antibodies

25 according to the invention may still have some binding affinity for the cell
surface antigen.

The ability of the antibody according to the invention to
induce immunological tolerance to a therapeutic cell-binding antibody relies
on the presence in the non-cell-binding antibody of at least one epitope

30 also present in the therapeutic antibody, which induces an immune

response in the intended patient.

The non-cell-binding antibody is preferably capable of tolerising to anti-idiotypic responses, at least to the V domain hypervariable regions of the therapeutic antibody and preferably also to the framework regions. Thus it is desirable that the tolerising antibody has an amino acid sequence similar to the therapeutic antibody in those regions. Preferably there is >90%, or >95% or >99% amino acid sequence identity between the variable domains of the non-cell-binding antibody and the therapeutic antibody. Most preferably the differences are restricted to any amino acid substitution(s) required to sufficiently reduce antigen binding affinity in the non-cell-binding antibody.

Preferably also the non-cell-binding antibody is capable of inducing tolerance to the constant regions of the therapeutic antibody. Thus, it is preferred that the constant domains of the non-cell-binding antibody are similar to those of the therapeutic antibody, having for example >90% or >95% or >99% amino acid sequence identity. Most preferably, the constant domains of the non-cell-binding antibody and the therapeutic antibody are identical and are thus matched allotypically.

The invention further provides fragments of an antibody described herein, the fragments having tolerance-inducing capability. Such fragments include monovalent and divalent fragments such as Fab, Fab· and F(ab·)₂ fragments. Also included are single chain antibodies. The preferred features of such fragments are as described herein in relation to non-cell-binding antibodies according to the invention. The non-cell-binding fragments may be for use with corresponding therapeutic antibody fragments, or with therapeutic antibody molecules.

The reduced binding affinity of the non-cell-binding antibodies may be achieved in a variety of ways. In the preferred embodiment described herein, an alteration in the CDRs comprising one or two or more amino acid substitutions reduces binding affinity. Alternatively, amino acid

substitutions in other parts of the antibody molecule may be used to reduce
binding affinity. For example, amino acid substitutions in the framework
regions are known to significantly affect binding affinity (Reichmann *et al*
1988). Another alternative is a monovalent form of the therapeutic

5   antibody. Monovalent antibodies have reduced binding affinity compared
to their bivalent counterparts. Monovalent forms may be for example Fab
fragments, or single chain antibodies, or any other genetically engineered
antibody fragments retaining a single binding site. Monovalent variants can
also be produced by mutating the cysteine residue which participates in

10   interchain (H-H) disulphide formation (e.g., cys → ser or cys →ala). The
reduction in binding affinity of a monovalent antibody compared to its
bivalent counterpart may be sufficient to enable tolerance induction.
Preferably, the monovalent antibody is either incapable of binding Fc
receptors, or incapable of binding complement component C1q, or both.

15   Either or both of these properties can be introduced by suitable mutations
(see e.g., Morgan *et al.*, WO 94/29351, published 22 December 1994 and
Winter *et al.*,EP 0 307 434 B1).

        The non-cell-binding antibodies or fragments according to the
invention may thus be one of a variety of types of antibodies or fragments,

20   including genetically engineered antibodies or antibody fragments. In
addition, the antibodies or fragments will generally be from a mixture of
origins. For example, they may be chimeric e.g. human constant regions
with rat variable domains; or humanised or CDR grafted or otherwise
reshaped (see, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*,

25   European Patent No. 0 125 023 B1; Boss *et al.*, U.S. Patent No.
4,816,397; Boss *et al.*, European Patent No. 0 120 694 B1; Neuberger,
M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No.
0 194 276 B1; Winter, U.S. Patent No. 5 225 539; Winter, European Patent
No. 0 239 400 B1; Queen *et al.*, U.S. Patent No. 5,585,089; Queen *et al.*,

European Patent No. 0 451 216 B1; Adair et al., WO 91/09967, published 11 July 1991; Adair et al., European Patent No. 0 460 167 B1; and Padlan, E.A. et al., European Patent No. 0 519 596 A1. See also, Newman, R. et al., Biotechnology, 10: 1455-1460 (1992), regarding primatized antibody,

5   and Huston et al., U.S. Patent No. 5,091,513; Huston et al, U.S. Patent No. 5,132,405; Ladner et al., U.S. Patent No. 4,946,778 and Bird, R.E. et al., Science, 242: 423-426 (1988) regarding single chain antibodies). Campath-1H is considered humanised although it contains two amino acid substitutions in the framework regions.

10   Ideally, the antibody according to the invention is as close as possible to the therapeutic antibody on which it is based. Administration of such a "minimal mutant" prior to injection of the cell-binding therapeutic mAb can be used to tolerise to all T- and most B-cell epitopes in the therapeutic mAb. Classic experiments indicate that tolerance is maintained

15   more effectively by T- cells than by B-cells. But since most B-cell responses including the anti-Id response require T-cell help, even if a B-cell is responsive to a given antigen, antibody production will be determined by the state of responsiveness of the T-cells (Chiller et al, 1971). Thus, it will be preferable to use a non-cell-binding variant which

20   contains the minimum differences needed to reduce its affinity for the cell-surface antigen sufficiently to enable it to be used as a tolerogen. By using techniques such as X-ray crystallography, computer modeling and site-directed mutagenesis, and also genetic methods such as phage display, it will be possible to design suitable non-cell-binding variants for any cell-

25   binding therapeutic antibody.

The antibody according to the invention is preferably in biologically pure form, desirably being at least 95% (by weight) free of other biological materials.

As used herein, the term "cell-surface antigen" means an

30   antigen which is found on cell surfaces, but not necessarily exclusively on

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cell surfaces.

The term "therapeutic antibody" is used herein to refer to an antibody which may be administered to humans or animals to have a desired effect in particular in the treatment of disease. Such therapeutic antibodies will generally be monoclonal antibodies and will generally have been genetically engineered.

In another aspect the invention comprises a composition for administration to a patient comprising an antibody as described herein, together with a physiologically acceptable diluent or carrier.

In a further aspect, the invention provides a host cell or cell line which expresses an antibody as herein described and use of such a host cell or cell line for the production of such an antibody.

Additional aspects of the invention include the use of an antibody as described herein in the manufacture of medicament for the induction of tolerance, in particular tolerance to a therapeutic antibody.

In attached figures:

Figure 1 shows the Campath-1H heavy chain minimal mutant constructs prepared as described in the Examples.

Figure 2 shows the PCR mutagenesis strategy for preparing the mutant constructs of Figure 1.

Figure 3 shows pGEM9zf containing wild type Campath-1H heavy chain, and substitution of mutant fragments in the heavy chain.

Figure 4 shows a schematic representation of one embodiment of a monovalent non-cell-binding therapeutic antibody.

Using rational design to create a "minimal mutant"

In one embodiment for producing a non-cell-binding variant of a therapeutic mAb, amino acid residues which are involved in binding to target antigen are identified. Relative to the number of residues that comprise the VL and VH domains, those that are directly involved in

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interactions with antigen are small in number (Novotny et al, 1983). And although the Ab-combining site is made up of 6 hypervariable loops, 1 or 2 of those loops may dominate in that interaction. If a key residue or residues can be identified, it/they can be changed by site-directed

5   mutagenesis to a residue that will reduce (reduce or abolish) antigen-binding. Because these residues will most likely be found within the hypervariable loop structures and not in the framework sequence supporting those loops, small changes may not significantly disrupt the overall structure of the Ab.

10

Model building of Ag-binding sites to define key residues for mutagenesis:

Because the constant regions and variable regions of Ab molecules are very similar in sequences and structures, general principles regarding Ab structure have been defined using relatively few solved

15   crystal structures. To date, approximately 50 structures of Ab fragments have been included in the Brookhaven Protein Data Bank, and of these, 20 % have been refined to a resolution of 2.0 angstroms or better. As the structural knowledge base increases, comparative Ab modelling (modelling by homology) becomes more reliable since there is a greater choice of

20   structural templates. Variable regions (VL and VH) of different Ab structures can be combined as a structural template after superimposing their most conserved residues. Side chain conformations of buried residues are then modelled. The CDR loops are modelled by identification of structurally similar loop templates (often loops with the same length and

25   similar sequence have similar backbone conformations). These CDR sequences often fall into canonical loop motifs (excluding H3, between 50 and 95 % of murine VL (kappa) and VH have loop sequences consistent with classified canonical motifs). Canonical loop backbones can then be spliced onto the model of the framework and CDR side-chain

30   conformations can be modelled based on conformations of residues found

at corresponding positions in other loops of the same canonical structure. Finally the model is often refined using computer programs that minimize troublesome stereochemical constraints.

Comparative model building is becoming widely used as the
5   size of the structural database increases, providing a greater range of structural templates. Also, the greater range of computer programs available ensures that models are becoming increasingly accurate. For example, the solved crystal structure of Campath-1H was very close to the structure predicted by molecular modelling. We could predict from the
10   modelling data that mutations 1 and 2 described in the Examples were likely to have a detrimental effect on binding to CD52. The crystal structure confirmed these predictions and also predicted that mutation 3 could disrupt binding to CD52.

Obviously, to create a non-cell-binding version of a
15   therapeutic Ab, it is desirable to start with a solved crystal structure, preferably co-crystallized with antigen so that the key contact residue(s) can be identified and substituted for residue(s) that destroy antigen binding. However, in many cases, a good molecular model could provide the necessary information. In cases where the molecular model is of poor
20   quality (for example, if the appropriate structural templates do not exist in the databank), CDR swapping experiments (as described in the Examples) will provide information on which CDRs must be targeted for mutation. Alanine scanning mutagenesis (mutating each residue sequentially to Ala) through those regions could identify the key residue(s) involved in antigen-
25   binding (Cunningham and Wells, 1989). If changing a single residue to Ala reduced but did not destroy binding, that position could be targetted for more drastic mutations (for example, a substitution that created in a charge difference) to further reduce binding, if desired.

Alternative methods for obtaining non-cell-binding versions of
30   therapeutic antibodies include genetic techniques such as phage display

using error prone PCR (Gram *et al*, 1992) and cycling of V-region genes (e.g. as sFv constructs) through a bacterial mutator strain (e.g. mutD5) (Low *et al.*, 1996). Such genetic methods can provide powerful screening systems.

The invention will now be further described in the examples which follow. Although the specific example of Campath-1H is given in this document, the invention is not limited to antibodies based on Campath-1H. It is anticipated that other cell-binding therapeutic antibodies, especially those which would be given in repeated doses, will become more widely accepted using this strategy.

EXAMPLES

A. Creating a "minimal mutant"

We have devised a method to determine which of the CDR loops of the humanized Campath-1 mAb are the most important ones for binding to CD52. Mutant VL or VH were genetically constructed in which each of the 6 hypervariable regions (as defined by Kabat *et al* (1987) using amino acid sequence alignments of V-regions in the protein databases) was individually swapped for the corresponding CDR from the V-region that had provided the human VL or VH acceptor sequence during humanization (REI and NEW, respectively). The engineered V-regions were expressed as Fab fragments in E coli using the pHEN vector (Hoogenboom et al, 1991). In this system, the pelB leader sequence was used to direct protein expression to the periplasm, where association of L-chain and truncated H-chain occurs (Hoogenboom et al, 1991). When these Fab fragments were assayed for binding to immobilized CD52, it was found that swapping the (VH) CDR2 of NEW into the humanized Campath-1 Fab completely destroyed binding to CD52. Replacing (VH) CDR3 reduced binding to CD52 8-fold while replacing (VH) CDR1 and (VL) CDR3 reduced binding 3-fold. No change in binding was detected when (VL)

CDR1 or (VL) CDR2 were replaced. From these results, it appeared that
(VH) CDR2 contained a key residue(s) necessary for antigen-binding.
DNA encoding the "wild-type" humanized Campath-1 H-chain (Reichmann
et al, 1988) was used as PCR template for site-directed mutagenesis. This

5   heavy chain sequence encodes human protein at all positions except the
three VH CDR regions and positions 27 and 30 of the first framework
region. We focussed on the H2 loop within VH CDR2 to make mutations
which would abolish binding of the Ab to CD52. H2 is the actual loop
structure (Chothia and Lesk, 1987) that is found within the 19 amino acid

10   VH CDR2 denoted "hypervariable" by Kabat et al's definition (Kabat et al,
1987) (see Figure 1). It is known that a few key residues in the loop and /
or framework regions determine relatively few CDR loop conformations and
canonical loop motifs have been identified for most CDR including VH
CDR2 (Chothia and Lesk, 1987). Since it is the loop structures that stick

15   out from the V-region β-barrel framework to make contact with antigen,
mutations in the loop would have the greatest chance of destroying antigen
binding whilst preserving Ab structure. In general, we restricted the
changes to the H2 loop except for H-chain mut6 which contained an
additional mutation in the residue immediately preceeding H2 as discussed

20   in more detail below.

Summary of Campath-1H heavy chain minimal mutant constructs (Figure 1)
                Mutation 1 is a single charge difference at residue 52b from

25   Lys to Asp. It was predicted from the molecular modelling of Campath-1H
Ab, and supported by the crystal structure, that the side chain of this
residue is pointing out of the Ag binding pocket, towards the approach of
antigen. Since the positive charge of the Lys is thought to interact with the
negatively charged phosphate groups of the GPI anchor of CD52, it is

30   possible that this single mutation will destroy antigen-binding.

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Mutation 2 is a single charge difference at residue 52a from Asp to Lys. It was predicted from the molecular modelling of Campath-1H Ab that this change could interfere with antigen-binding.

Mutation 3 is a single charge difference at residue 53 from Lys to Asp. From the crystal structure of Campath-1H Ab, it is clear that the majority of this residue side chain is solvent accessible and therefore may be involved in the interaction with the negatively charged phosphate groups of the GPI anchor of CD52, as for mutation 1.

Mutation 4 is a double mutation encompassing the individual substitutions of mutant 1 and 3 (Lys52b and Lys53 to Asp).

Mutation 5 is a triple mutation encompassing the individual substitutions of mutant 1, 2 and 3 (three charge differences: Asp52a to Lys; Lys52b and Lys53 to Asp).

Mutation 6 is a triple mutation encompassing the individual substitutions of mutant 1 and 2 (two charge differences Asp52a to Lys; Lys52b to Asp), and an additional mutation of Arg52 to Ala. Residue 52 has been shown to differ between 3 different Campath-1 Ab of high, low and moderate affinity and may be therefore directly involved in affinity maturation. This in turn might suggest a role in antigen binding.

For each of these heavy chain mutations, the change(s) was/were encoded on oligonucleotide primers 1B and 2A (figure 2). PCR was carried out on "wild-type" Campath-1 heavy chain DNA using a 5' primer annealing to the leader sequence and containing an upstream HindIII site (primer 1A) and primer 1B to generate a 200 bp fragment. Similarly, primer 2B (annealing to CH1 and containing a BstXI site followed by an EcoRI site) and primer 2A, a 440 bp fragment was generated. These fragments were gel purified and then combined in a single PCR reaction. Primer 1A and primer 2B were added after the first cycle (thus allowing the 2 pieces of overlapping DNA to anneal before amplification). Following PCR, the fragments were gel purified and digested with HindIII and EcoRI

and were transferred into intermediate sequencing vectors (PUC19 or pGEM3zf) for verification of sequence. A unique PstI site located in the Campath-1H VH and a unique BstXI site in the CH1 region allowed the mutant V-regions (and partial CH1 sequence) to be isolated as PstI-BstXI

5 fragments such that the mutation(s) were encoded in six different DNA cassettes flanked by PstI and BstXI sites. To create the mutant Campath-1H heavy chains, the original heavy chain construct (in intermediate vector pGEM9zf) was cut with PstI and BstXI and the fragment was removed. The remaining DNA (encoding the Campath-1H heavy chain leader sequence

10 and the V-region upsteam of the PstI site, plus the CH1 region downstream of the BstXI site followed by the hinge, CH2, CH3 in pGEM9zf) was gel purified (figure 3). Ligations were then set up in which each of the DNA cassettes containing the mutation(s) described above was joined to the gel purified pGEM9zf/PstI-BstXI cut Campath-1H heavy chain DNA. These six

15 mutagenized Campath-1H heavy chains were then isolated by digestion with HindIII and gel purification, followed by ligation into HindIII cut mammalian expression vector pBAN-2. This vector is derived from the pNH316 vector that contains a neomycin selectable marker under the control of the mouse metallothionein promoter and the strong human

20 β-actin promoter/polyadenylation signals for expression of the desired gene product (Page and Sydenham, 1991). As these fragments were introduced into a single HindIII restriction site, orientation of each fragment was checked by DNA sequencing.

25 <u>Campath-1H light chain construct for co-transfection:</u>

DNA encoding the humanized "wild type" Campath-1H light chain (human sequence at all residues except the three CDR in the V-region) was isolated from an intermediate vector as a HindIII to EcoRI fragment. This fragment was gel purified and then ligated into HindIII-

30 EcoRI cut mammalian expression vector pRDN-1. This vector is derived

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from the pLD9 vector that contains a "crippled" dihydrofolate reductase
(dhfr) selectable marker (the enhancer element of the SV40 promoter has
been removed to allow for increased levels of gene expression in the
presence of methotrexate) and the strong human β-actin
5   promoter/polyadenylation signals for expression of the desired gene
product (Page and Sydenham, 1991).

Co-transfection of Campath-1H light chain DNA and mutant heavy chain DNA:

10   The expression system used to produce high levels of
humanized Campath-1H Ab in the past is the widely used mammalian
expression system featuring gene amplification by the use of dihydrofolate
reductase (dhfr) deficient Chinese hamster ovary (CHO) cells and the use
of strong β-actin promoters for selection and amplification of the desired
15   gene products (Page and Sydenham, 1991).

The following transfections (TF) were carried out:

      TF1:   mock ("empty" pRDN-1 plus "empty" pBAN-2)

      TF2:   Light chain only (Light-chain/pRDN-1 plus "empty"
20   pBAN-2)

      TF3:   Light chain/pRDN-1 plus H chain mutant 1/pBAN-2

      TF4:   Light chain/pRDN-1 plus H chain mutant 2/pBAN-2

      TF5:   Light chain/pRDN-1 plus H chain mutant 3/pBAN-2

      TF6:   Light chain/pRDN-1 plus H chain mutant 4/pBAN-2

25   TF7:   Light chain/pRDN-1 plus H chain mutant 5/pBAN-2

      TF8:   Light chain/pRDN-1 plus H chain mutant 6/pBAN-2

      TF9:   Light chain/pRDN-1 plus H chain "wild-type"/pBAN-2

DNA (20 μg of light chain/pRDN-1 plus 20 μg of heavy chain/pBAN-2) was mixed in a sterile eppendorf, ethanol precipitated, and rinsed twice with 70% ethanol. DNA pellets were resuspended in sterile Tris-EDTA.

5   For each transfection, the DNA was diluted with 60 μl of 20 mM HEPES (pH 7.4) in a 5 ml polystyrene tube. In another tube, 120 μl of DOTAP liposomal transfection reagent (Boehringer Mannheim) was diluted with 80 μl of 20 mM HEPES (pH 7.4). Then the DNA/HEPES was added to the diluted DOTAP, mixed gently, and left at room temperature for 15 min. Culture medium (IMDM + 5% FCS + HT) was aspirated from a T75 flask

10   containing dhfr deficient CHO cells growing at approximately 50% confluency. The DNA/DOTAP was then added to the flask along with 10 ml fresh culture medium. The flask was cultured for 24 h at 37°C in 5% CO_2. The DNA/DOTAP was then aspirated from the flask and the cells were given 15 ml fresh culture medium. After a further 24 h, selection was

15   initiated by removing the culture medium and adding selection medium (IMDM + 5% dialysed FCS + 1 mg/ml G418). The cells were cultured at 37°C in 5% CO_2 and fresh selection medium was added as necessary. Culture supernatants were then tested by ELISA for the presence of antibody as described below.

20

Detection of secreted Ab in transfection supernatants by ELISA:

          Microtitre plates were coated with 50 μl/well anti-human Ig Fc (Sigma, catalogue number I-2136) in PBS at 2.5 μg/ml overnight at 4°C. The coating Ab was removed and the plates were blocked by addition of

25   100 μl/well blocking buffer (PBS + 1% BSA + 5% FCS + 1% heat-inactivated normal rabbit serum (NRS) overnight at 4°C. The transfection supernatants were added (50 μl/well) for at least 1h at room temperature. The wells were washed with PBS/0.5% Tween-20 (PBS/Tween). Biotinylated sheep anti-human Ig (Amersham, catalogue number RPN

30   1003) diluted 1/5000 in blocking buffer or biotinylated goat anti-human

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kappa light chain (Sigma, catalogue number B-1393) diluted 1/1000 in blocking buffer was added (50 μl/well) for 1h at room temperature. The wells were washed with PBS/Tween and 50 μl/well ExtrAvidin-peroxidase (Sigma, catalogue number E-2886) was added for 30 min at room

5   temperature. The wells were washed once more and 100 μl/well of substrate o-phenylenediamine dihydrochloride (Sigma, catalogue number P-7288) was added. Colour change was measured at 492 nM using a Multiskan Plus microtitre plate reader.

10   Detection of binding to Campath-1 antigen by ELISA:

Microtitre plates were coated with 50 μl/well anti-mouse Ig Fc (Sigma, catalogue number M-4280) in PBS at 2.5 μg/ml overnight at 4°C. The coating Ab was removed and the plates were blocked by addition of 100 μl/well blocking buffer (PBS + 1% BSA + 5% FCS + 1% heat-

15   inactivated NRS) overnight at 4°C. Purified recombinant Campath-1 Ag-fusion protein (sequence encoding the CD52 peptide backbone fused to sequence encoding mouse CH2 and CH3 domains, and purified on a protein A column) was then added at 4 μg/ml to each well (50 μl/well) in PBS overnight at 4°C. The wells were then washed with PBS/Tween and

20   the transfection supernatants were added (50 μl/well) for at least 1h at room temperature. The wells were washed withPBS/Tween and biotinylated sheep anti-human Ig (Amersham, catalogue number RPN 1003) diluted 1/5000 in blocking buffer was added (50 μl/well) for 1h at room temperature. The wells were washed with PBS/Tween and 50 μl/well

25   ExtrAvidin-peroxidase (Sigma, catalogue number E-2886) was added for 30 min at room temperature. The wells were washed once more and 100 μl/well of substrate o-phenylenediamine dihydrochloride (Sigma, catalogue number P-7288) was added. Colour change was measured at 492 nM.

30

22

Assessment of non-binding mutants by ELISA:

"Wild-type" purified Campath-1H Ab binds strongly to recombinant Campath-1 Ag-fusion protein in ELISA assays. To provide a comparison for assessing non-cell-binding antibodies, the wild-type Ab can be titrated down in concentration until binding is just detectable. This may be referred to as "1 Ab binding unit". A suitable non-binding mutant of the wild-type will not show detectable binding at many times this concentration e.g. 100 times, or 1000 times, or preferably 10,000 times this concentration of wild-type Campath-1H Ab.

Assessment of non-binding mutants *in vivo*:

An alternative method which can be applied to assess non-binding potential is described. Because we know that the wild-type Campath-1H Ab elicits a strong anti-immunoglobulin response in transgenic mice expressing human CD52 (see next section for details on these mice), purified deaggregated preparations of the mutants can be used *in vivo* to assess whether they are immunogenic. If an anti-globulin response cannot be detected at doses between 1μg and 1mg deaggregated mutant per mouse, this is a good indication that the mutant is unable to bind CD52.

B. In vivo models of tolerance induction:

To test the ability of the minimal mutants of Campath-1H to tolerize to the wild-type Campath-1H Ab in vivo, transgenic mice are used. For example, transgenic mice expressing human CD52 behind a murine CD2 promoter to mimic the expression of CD52 on T-cells can be used.

To create such mice, a 2.8 kb genomic fragment containing the 2 exons of the human CD52 gene as well as 4.5 kb upstream and 3' flanking sequence of the human CD2 gene can be introduced into the genome of transgenic mice. It is thought that strong control regions are present 3' to

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the human CD2 gene that determine the high levels and tissue-specific
expression of the gene (Greaves *et al*, 1989). By this method, four
CD52/CBA founders were established that transmitted the transgene.
Indeed, when peripheral blood staining of their offspring was analysed by
5   fluorescence activated cell sorting and 2-colour staining, it was shown that
the cells expressing mouse CD3 (T-cells) also expressed human CD52 (D.
Kioussis, unpublished data). These mice were bred to homozygosity and
greater than 95% of their T-cells express high levels of human CD52 on
the cell surface.

10   These mice produce a vigorous anti-globulin response (titre of
1/1000 or greater) to wild-type Campath-1H at doses of 1 to 10 mg/mouse.
This anti-globulin response includes an anti-Id component as the CDR
loops are rat sequence. The effectiveness of the minimal mutants to
tolerize to subsequent challenge of wild-type Campath-1H Ab may be
15   assessed in the following ways:

1.   Intravenous administration of a single dose (0.5 to 1 mg/mouse day
0) of each non-cell-binding mutant or irrelevant control Ab (deaggregated
by ultracentrifugation) followed by challenge with 1 to 10 mg wild-type
20   Campath-1H Ab at 4 to 6 wks. Tail bleeds 10 days post challenge are
tested by ELISA for Campath-1H anti-Id specificity.
2.   Intravenous administration of multiple doses (0.5 to 1 mg/mouse) of
deaggregated non-cell-binding mutant or control Ab over 2 months prior to
challenge with 1 to 10 mg wild-type Campath-1H Ab. Tail bleeds 10 days
25   post challenge are tested by ELISA for Campath-1H anti-Id specificity.
In both cases, the irrelevant control Ab is be an isotype-matched non-cell-
binding Ab in mice such as Campath-9 which is a humanized anti-CD4 Ab
(Gorman et al, 1991).

30

24

C. How the strategy could be adopted for human therapy:

An amount (e.g.500 mg) of the non-cell-binding form of the therapeutic antibody would be administered to a patient awaiting treatment with the therapeutic antibody. Preferably the non-cell-binding antibody as

5   administered is freshly deaggregated (for example by passage through a fine filter). A period of time later (for example 7 days), during which time the T-cells and B-cells would become tolerised, the wild-type form of the therapeutic antibody would be given.

1:). Additional considerations:

10   1.   It should be easier to tolerize to a minimal mutant than to HGG or to mixed chain Ab molecules.

In the tolerance models of Benjamin et al (1986), tolerance to polyclonal HGG was induced in mice following depletion of CD4+ T-cells, but also using deaggregated material. It was found that tolerance to these

15   soluble proteins could be achieved relatively easily. Also, in the work of Isaacs and Waldmann (1994), CD4 Ab were given during tolerance induction to the non-cell-binding mixed chain Ab molecules (irrelevant and antigen specific H- and L-chains), or non-cell-binding forms were used as tolerogens in their own right following their deaggregation.

20   In our modified approach to inducing tolerance using a minimal mutant, the foreignness of the protein will be less than that of polyclonal HGG or of the mixed chain Ab molecules in mice. In cases where the therapeutic mAb is humanized, only the CDR loops (and in some cases, some framework positions) are comprised of rodent

25   sequence. It therefore may be possible to tolerize with a deaggregated minimal mutant in the absence of CD4 mAb. However, even if CD4 administration was required, a humanized therapeutic CD4 is available (CAMPATH-9; Gorman et al, 1991). The studies in transgenic animals should address these details.

30   2. Creation of a monovalent form of the minimal mutant (Figure 4).

25

Thus far we have considered tolerance induction using a minimal mutant that is essentially like the wild-type therapeutic except for minimal residue change(s) that will disrupt antigen binding. We also propose a monovalent form that is also significantly smaller than the

5   minimal mutant.

In one embodiment, the monovalent form is a single-chain Fv [formed by the VL, a short peptide linker (such as those reviewed in Huston et al, 1991) and the mutated VH] genetically fused with the sequence encoding the hinge-CH2-CH3 of human IgG1. This construct is

10   expressed in association with a truncated heavy chain (hinge-CH2-CH3 only; Routledge et al, 1991) such that a protein is expressed that is composed essentially of a single Ab-combining site and a functional Ig Fc domain. The immunogenicity of the different peptide linkers is expected to be negligible given their small size (generally 14 to 18 residues in length)

15   and abundance of small residues (eg Gly and Ser) making up the linkers. A popular choice is the 15-residue linker (Gly$_4$Ser)$_3$ in which the serine residues confer extra hydrophilicity on the peptide backbone (to inhibit its intercalation between the variable domains during folding) and which is otherwise free of side chains that might complicate domain folding (Huston

20   et al, 1988).

SFv have been expressed in mammalian cells from a number of different antibodies and have been shown to fold into the correct conformation for antigen-binding by functional activity (Gilliland et al, 1996). The Fc portion is a preferred feature which should ensure serum half-life

25   comparable to the minimal mutant and to the wild-type therapeutic Ab, whilst monovalency will ensure that binding to CD52 is greatly reduced due to the decrease in avidity. We have already shown from the CDR-swapping experiments (section A1) that the Campath-1 Ab binds poorly to CD52 in a monovalent form. In addition to reducing the avidity of the

30   molecule, the smaller size may be a bonus: in classical tolerance

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experiments, it was found that the smaller the molecule, the better it was at inducing tolerance (Parish and Ada, 1969; Anderson, 1969; Miranda et al, 1973). By combining monovalency with a non-cell-binding mutant, a highly effective tolerogen may be obtained.

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Eur J Immunol 21, 1677-1684.

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CLAIMS

1.   An antibody which is a modified version of a therapeutic
antibody with affinity for a cell-surface antigen, said antibody having
reduced affinity for the antigen compared with the therapeutic antibody as
a result of a modification or modifications to the antibody molecule, wherein
the antibody is capable of inducing immunological tolerance to the
therapeutic antibody.

2.   An antibody as claimed in claim 1, wherein the framework
regions of the variable domains of the antibody have the same or
substantially the same amino acid sequence as the therapeutic antibody
framework regions.

3.   An antibody as claimed in claim 1 or claim 2, wherein the
modification comprises an alteration in at least one of the complementarity
determining regions (CDRs).

4.   An antibody as claimed in claim 3, wherein the alteration is
achieved by genetic manipulation of a nucleic acid coding for the CDR.

5.   An antibody as claimed in any one of claims 1 to 4, wherein
the affinity of the antibody for the antigen is reduced to 50% or less of the
affinity of the therapeutic antibody.

6.   An antibody as claimed in any one of claims 1 to 5, wherein
the CDRs are foreign with respect to the constant region of the antibody.

7.   An antibody as claimed in any one of claims 1 to 6, wherein
the CDRs are foreign with respect to the heavy and light chain variable
domain framework regions.

8.   An antibody as claimed in claim 7, which is of substantially
human origin other than the CDRs.

9.   An antibody as claimed in any one of claims 1 to 8, wherein
the therapeutic antibody has affinity for CD52.

10.   An antibody as claimed in claim 9, wherein the therapeutic antibody is a humanised Campath-1 antibody

11.   An antibody as claimed in claim 10, wherein the modification comprises an alteration in (VH) CDR2.

12.   An antibody as claimed in claim 11, wherein the modification comprises a single or a double amino acid substitution in (VH) CDR2.

13   An antibody as claimed in any one of claims 1 to 12, wherein the constant domains of the antibody have substantially the same amino acid sequence as the therapeutic antibody constant regions.

14.   A fragment of an antibody according to any one of claims 1 to 13, which fragment retains tolerance-inducing capability of the antibody.

15.   An antibody fragment as claimed in claim 14, which is a modified version of a therapeutic antibody fragment.

16.   An antibody or fragment as claimed in any one of claims 1 to 15, which is monovalent.

17.   An antibody or fragment as claimed in any one of claims 1 to 16, for inducing tolerance to the therapeutic antibody in a patient.

18.   A cell line which expresses an antibody or fragment as claimed in any one of claims 1 to 17.

19.   A method of producing an antibody which is a modified version of a therapeutic antibody with affinity for a cell-surface antigen, said antibody having reduced affinity for the antigen compared with the therapeutic antibody as a result of a modification or modifications to the antibody molecule, wherein the antibody is capable of inducing immunological tolerance to the therapeutic antibody, comprising maintaining a cell line as claimed in claim 18 under conditions suitable for expression of said antibody or fragment thereof.

20.   The method of claim 19, further comprising recovering the antibody or fragment.

21.   The method of claim 20, further comprising isolating the

antibody or fragment.

22.   A composition for administration to a patient, comprising an
. antibody or fragment as claimed in any one of claims 1 to 17 or as
produced by the method according to any one of claims 19 to 21, together
5   with a physiologically acceptable diluent or carrier.

23.   The use of an antibody or fragment as claimed in any one of
claims 1 to 17 or as produced by the method according to any one of
claims 19 to 21, in the manufacture of a medicament for the induction of
tolerance.

10

Figure 1

Campath-1H heavy chain mutations
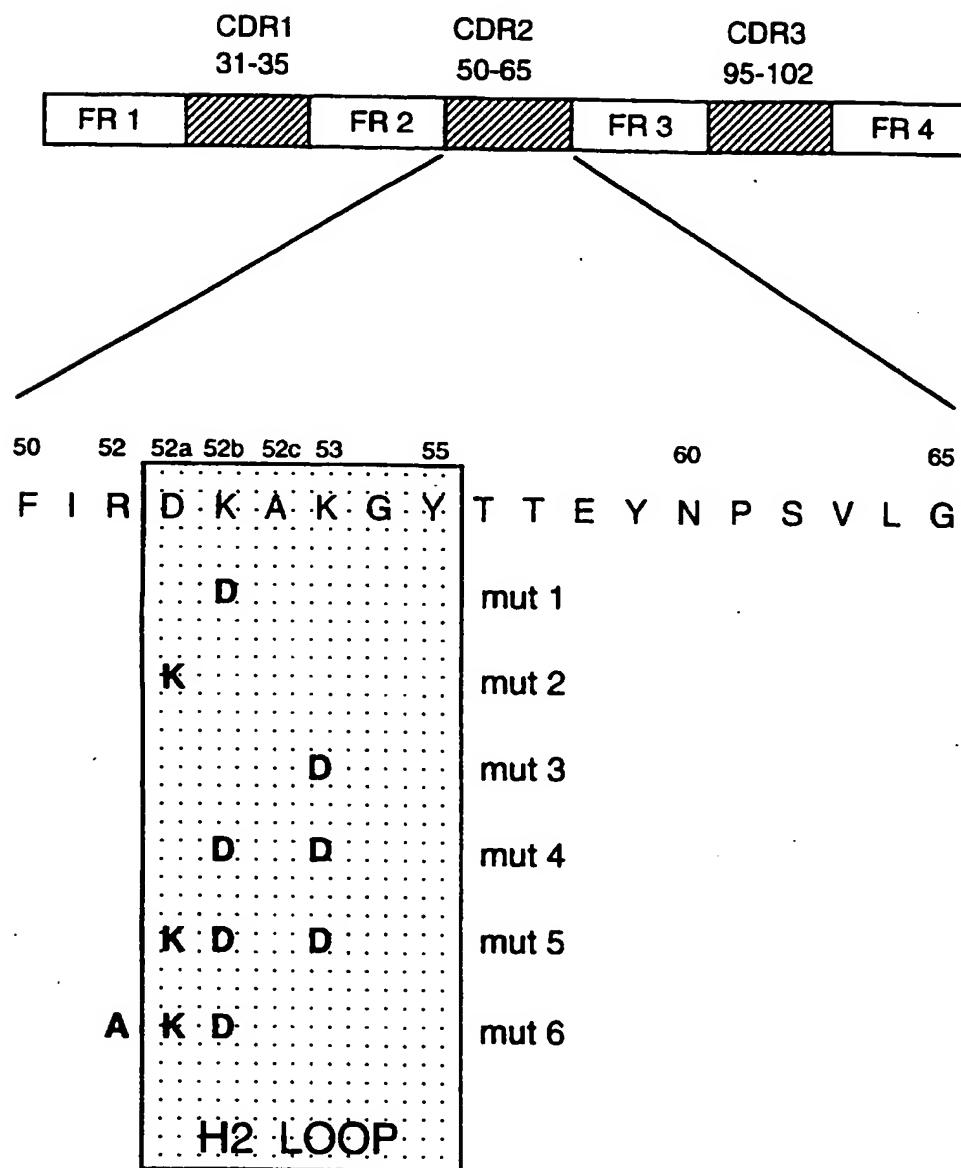
Figure 2

PCR mutagenesis strategy

purify fragments, combine

1 cycle with no PCR primers

add primers 1A and 2B and continue PCR cycling

gel purify fragment, digest, transfer to sequencing vector
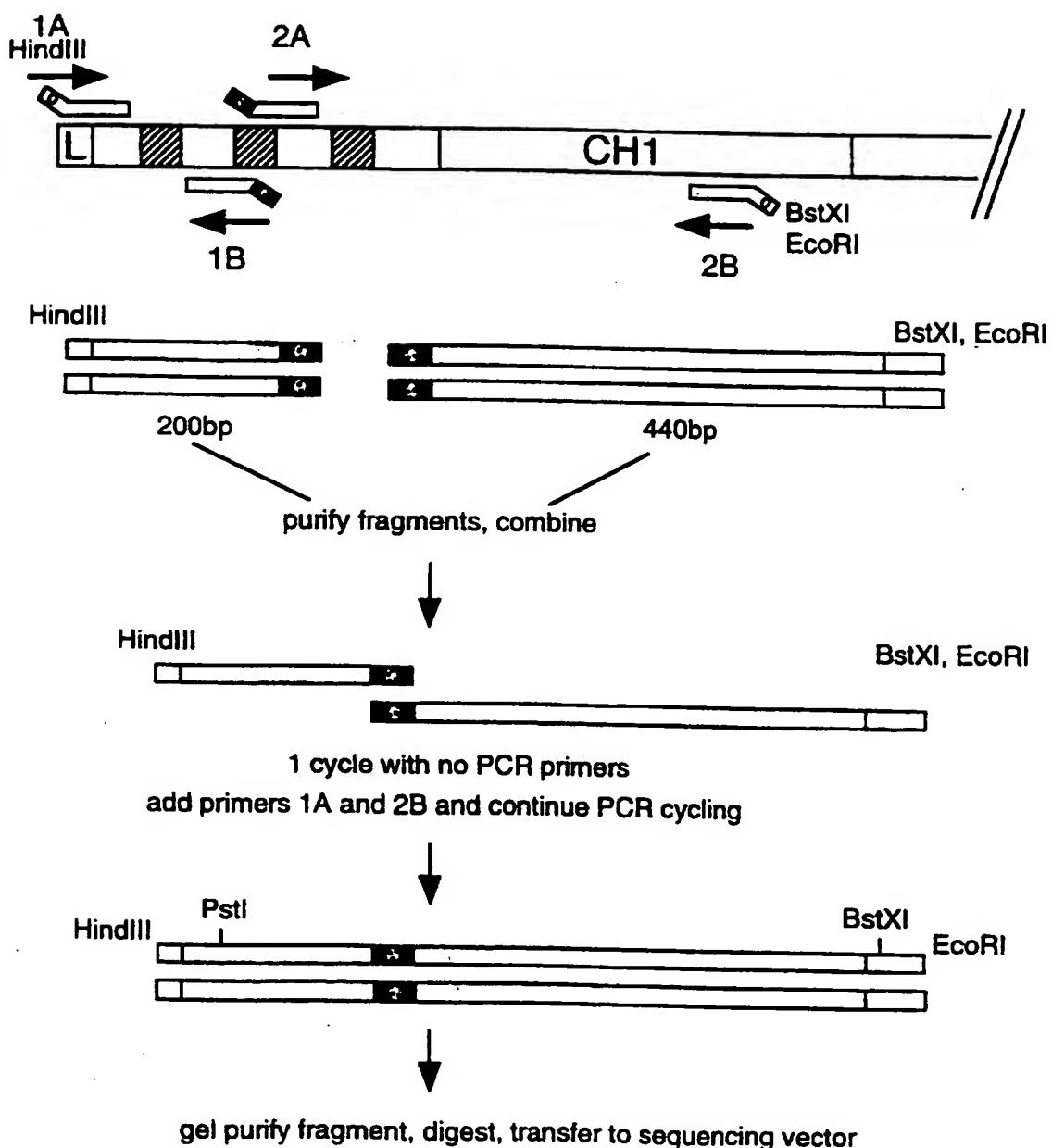
A. "Wild-type" Campath-1H heavy chain in pGEM9zf

remove PstI-BstXI fragment
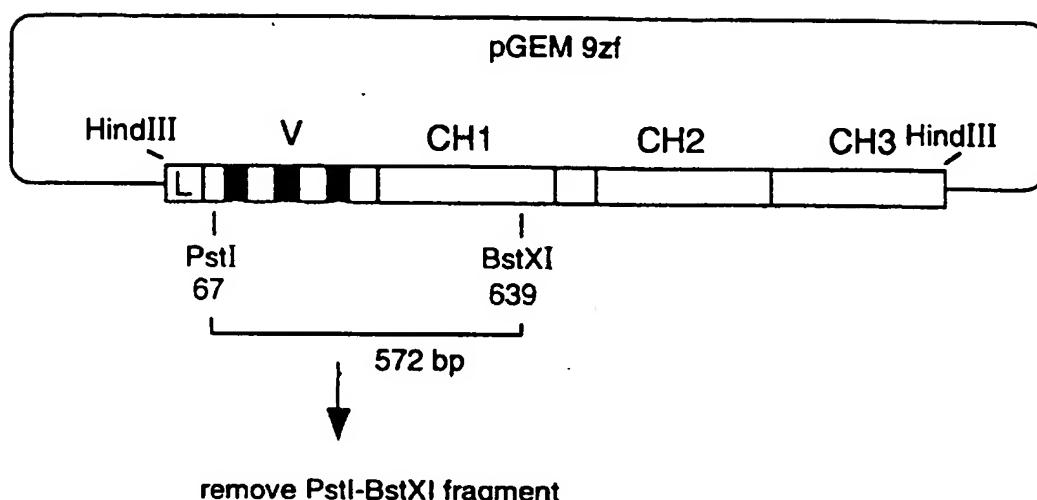
B. Substitute fragments carrying mutations

mutant fragments
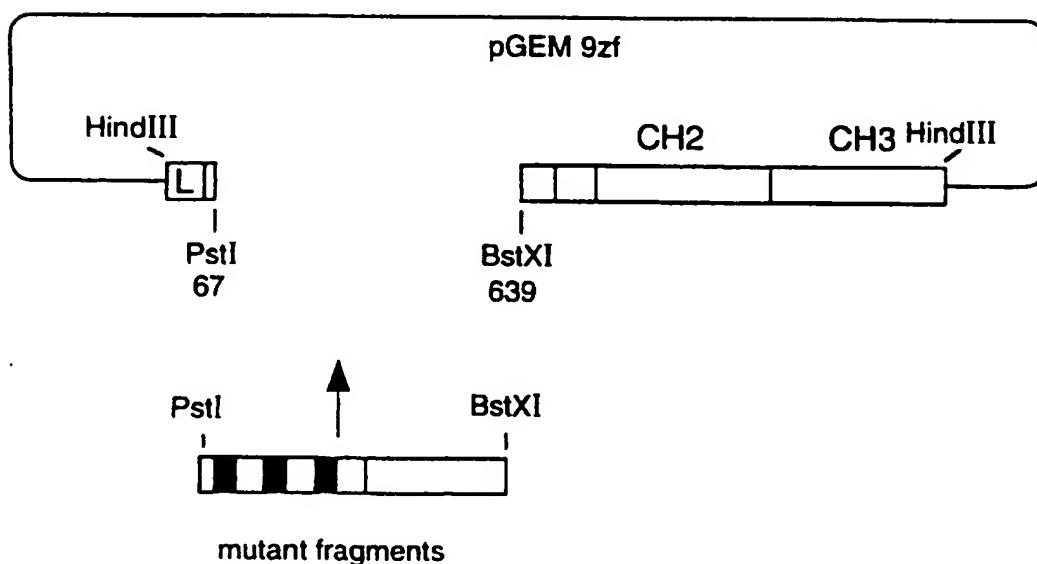
Figure 4

Schematic representation of a
monovalent sFv-Ig minimal mutant

VL

mutant
VH

LINKER

H

CH2

CH3

H

CH2

CH3
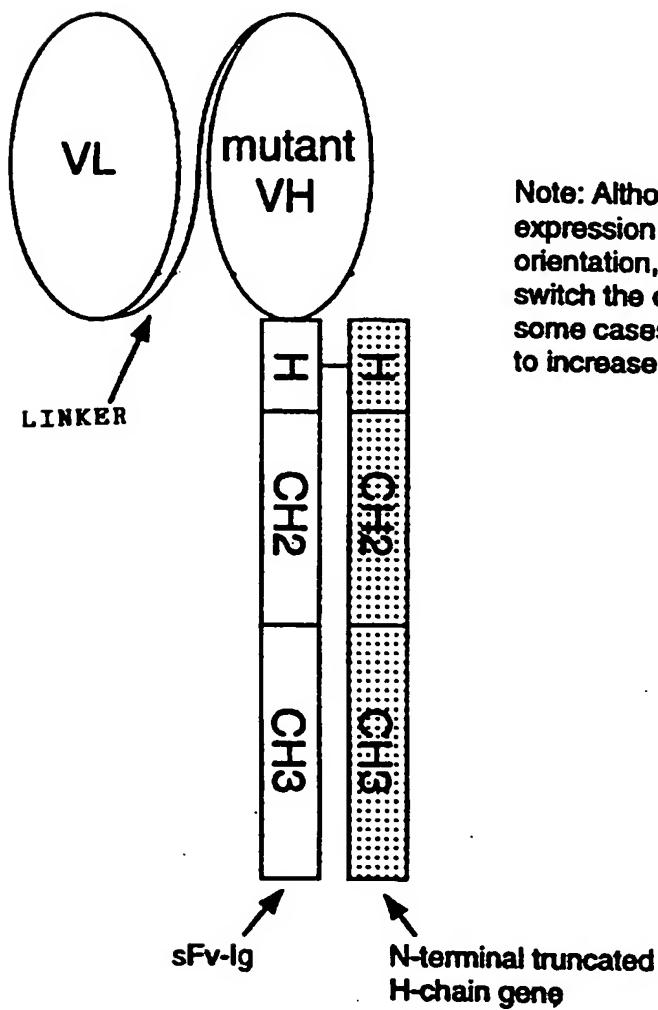
Note: Although this figure shows
expression of the sFv in the VL-VH
orientation, it is also possible to
switch the orientation to VH-VL. In
some cases this has been shown
to increase expression levels.

sFv-Ig

N-terminal truncated
H-chain gene

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07K16/28   C07K16/46   A61K39/395   C12N5/10   C12N15/13
         C12N15/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THERAPEUTIC IMMUNOLOGY, vol. 1, 1994, pages 303-312, XP000674792 ISAACS AND WALDMANN: "Helplessness as a strategy for avoiding antiglobulin responses to therapeutic monoclonal antibodies" cited in the application	1
Y	see the whole document --- -/--	2-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1997	07.07.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 8, 15 April 1992, pages 3576-3580, XP000384398 GRAM H ET AL: "IN VITRO SELECTION AND AFFINITY MATURATION OF ANTIBODIES FROM A NAIVE COMBINATORIAL IMMUNOGLOBULIN LIBRARY" cited in the application see the whole document ---	2-8, 13-23
Y	NATURE, vol. 332, March 1988, pages 323-327, XP002032968 RIECHMANN ET AL.: "Reshaping human antibodies for therapy" cited in the application	9-12
A	see the whole document ---	1-8, 13-23
A	J.EXP.MED., vol. 163, June 1986, pages 1539-1552, XP000674798 BENJAMIN ET AL.: "Tolerance to rat monoclonal antibodies" cited in the application see the whole document ---	1-23
A	NUCLEIC ACIDS RESEARCH, vol. 19, no. 15, June 1991, pages 4133-4137, XP002032967 HOOGENBOOM ET AL.: "Multi-subunit proteins on the surface of filamentous phage:methodologies for displaying antibody (Fab) heavy and light chains" cited in the application see the whole document ---	1-23
A	EP 0 328 404 A (MEDICAL RES COUNCIL ;WALDMANN HERMAN (GB); CLARK MICHAEL RONALD (G) 16 August 1989 cited in the application see the whole document ---	9-12,22, 23
A	WO 93 07899 A (WELLCOME FOUND) 29 April 1993 see the whole document ---	9-12,22, 23
A	WO 93 10817 A (HALE GEOFFREY ;WALDMANN HERMAN (GB)) 10 June 1993 see the whole document ---	9-12,22, 23

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	CLINICAL RESEARCH, vol. 38, no. 2, 1990, page c-235 XP002032969 LOCKWOOD ET AL.: "Monoclonal antibody therapy in systemic vasculitis" see the whole document ---	9-12,22, 23
A	ERLICH: "PCR Technology" 1990 , STOCKTON PRESS , US,NEW YORK XP002033036 166780 Higuchi: Using PCR to engineer DNA. Chapter 6, pages 61-70. see the whole document -----	9-12

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